

THE IDENTIFICATION AND MEASUREMENT OF SUGARS IN THE BLOOD OF THREE SPECIES OF ATLANTIC CRABS¹

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Although considerable attention has been given to the physiology and biochemistry of the Crustacea, their carbohydrate metabolism did not become known until Hu (1958) found evidence that it was not unlike that of other animals. Using paper chromatography Hu found glucose, maltose, maltotriose, maltotetrose and two other oligosaccharides in the blood of the crab, *Hemigrapsus nudus*. Since then these same sugars have been reported in *Cancer magister* (Meenakshi and Scheer, 1961) several other crabs (Dean and Vernberg, 1965a), the crayfish, *Orconectes virilis* (McWhinnie and Saller, 1960) and the lobster, *Homarus americanus* (Telford, 1968a). The non-reducing disaccharide, trehalose, was found in nine species of crustaceans by Fairbairn (1958) and in traces in some crabs (Dean and Vernberg, 1965a). In the American lobster trehalose appears to be a significant minor component of the blood involved in the response to handling stress (Telford, 1968b). Several monosaccharides, galactose, mannose, fucose and fructose were found with sporadic occurrence (above references).

In this paper the results of chromatographic analysis of the blood sugars in the crabs *Carcinus maenas*, *Cancer borealis* and *Cancer irroratus* are related to measurements of blood glucose and total reducing sugar. Some evidence of changes with the molt cycle and reproductive activity is also given. A comparison is then made between these data and the rather scattered observations in the literature.

MATERIALS AND METHODS

Collection of animals and blood samples

Both *C. borealis* and *C. irroratus* were obtained from lobster traps at Port Clyde Maine. Three point five to 4.0 ml. of blood was taken via the articular membrane at the base of the cheliped. Decapod crustaceans become hyperglycemic following the stress of handling (Abramowitz *et al.*, 1944). To avoid this reaction blood samples were obtained at the moment of capture with the animals in as nearly an undisturbed state as possible. *Carcinus maenas* was collected in the intertidal zone at Port Clyde. Only from the largest specimens could 4.0 ml. blood be obtained; normally only about 2.0 ml. were collected. The shore crabs, *C. maenas*, were obtained in several stages of the molt cycle but the other two species were taken only in premolt and late postmolt or intermolt because they do not enter the traps at other times. Collections were made monthly from May

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through October, 1966, and September, 1967. The size range of *C. borealis* used was from 6 cm. up to about 18 cm. across the carapace. *Cancer irroratus* is smaller and the range used was 5–15 cm. The size range of *C. maenas* was 4–11 cm. In all three species males are generally larger than females.

As anticoagulant 4.0 ml. Heller's oxalate (Gradwohl, 1943) was used (2.0 ml. for the smaller blood samples). The anticoagulant was evaporated to dryness before use, thus avoiding dilution of the blood sample. The oxalated blood was stored frozen until use.

Preparation of samples

Large amounts of oxalate are necessary because coagulability varies during the molt cycle, presumably because of fluctuating blood calcium levels (Travis, 1955). Excess oxalate, however, interferes with the determination of glucose. Before deproteinizing the 1.0-ml. aliquots of blood, oxalates were precipitated by addition of 2.0 ml. 1% CaCl_2 . Proteins were precipitated by the Somogyi (1930)

TABLE I

Monthly levels of blood glucose and reducing substances (RS) in Cancer borealis (mg./100 ml.) Port Clyde, Maine, May–October, 1966

| Month | ♂ N | Glucose | (S.E.) | R.S. | (S.E.) | ♀ N | Glucose | (S.E.) | R.S. | (S.E.) |
|------------------|--------|---------|--------|------|--------|--------|---------|--------|------|--------|
| May | 48 | 6.8 | (0.49) | 10.5 | (0.55) | 49 | 8.0 | (0.62) | 10.0 | (0.57) |
| June | 47 | 7.7 | (0.38) | 12.2 | (0.51) | 43 | 6.6 | (0.47) | 10.3 | (0.54) |
| July | 50 | 8.0 | (0.36) | 10.5 | (0.45) | 43 | 7.6 | (0.25) | 9.4 | (0.30) |
| August | 51 | 10.6 | (0.47) | 15.3 | (0.55) | 52 | 8.4 | (0.50) | 11.1 | (0.57) |
| September | 53 | 10.2 | (0.69) | 12.4 | (0.74) | 53 | 10.2 | (0.57) | 12.8 | (0.63) |
| October | 43 | 9.9 | (0.46) | 12.9 | (0.52) | 52 | 9.5 | (0.45) | 12.1 | (0.48) |
| September (1967) | 58 | 8.7 | (0.47) | 13.1 | (0.81) | 64 | 8.8 | (0.43) | 13.0 | (0.67) |

method using 2.0 ml. 0.6% NaOH followed by 2.0 ml. 2.2% ZnSO_4 (acidified by drop-wise addition of conc. H_2SO_4 so that 1 vol. of zinc sulfate solution exactly neutralized 1 vol. of 0.6% NaOH). After centrifuging a clear supernatant was obtained, representing a 1:7 dilution of the blood. A 1.0-ml. aliquot of this supernatant was diluted 1:4 with distilled water.

Determination of glucose and reducing substances

Glucose was determined in 1.0-ml. duplicates of the original Somogyi supernatant using glucose oxidase-o-diansidine ("Glucostat," Worthington Biochemical Corp., Freehold, N. J.) dissolved in 0.02 M phosphate buffer, pH 7.0; further details of this procedure have appeared elsewhere (Telford, 1965). Reducing substances were determined in 1.0-ml. duplicates of the diluted Somogyi supernatant (above) using the Folin-Malmros alkaline potassium ferricyanide method (Dische, 1962). In both cases a blank of distilled water and three glucose standards of 5, 10, 20 mg./100 ml. were prepared in exactly the same way as the blood whenever determinations were made.

Paper chromatography

In preparation for chromatography individual blood samples of 1.0 or 2.0 ml. (as available) were coagulated by brief immersion in a boiling water bath (about 45 sec.). The clot was broken up and extracted with three washings of 60% methanol which were then pooled. Deionization on ion exchange columns followed (Telford, 1965) and the eluate was dried at 40° C. The residue was redissolved in 0.1 or 0.2 ml. pyridine (depending on size of original blood sample). Various sugars, alone and in mixtures, were prepared in the same way without showing any changes in chromatographic characteristics. The pyridine solutions were spotted onto Whatman #3 paper and developed for 16 hr. in a descending flow of ethyl acetate:pyridine:water, 8:2:1 (v/v/v) (Jermyn and Isherwood, 1949).

Detection of sugars on the developed chromatograms was by one of three

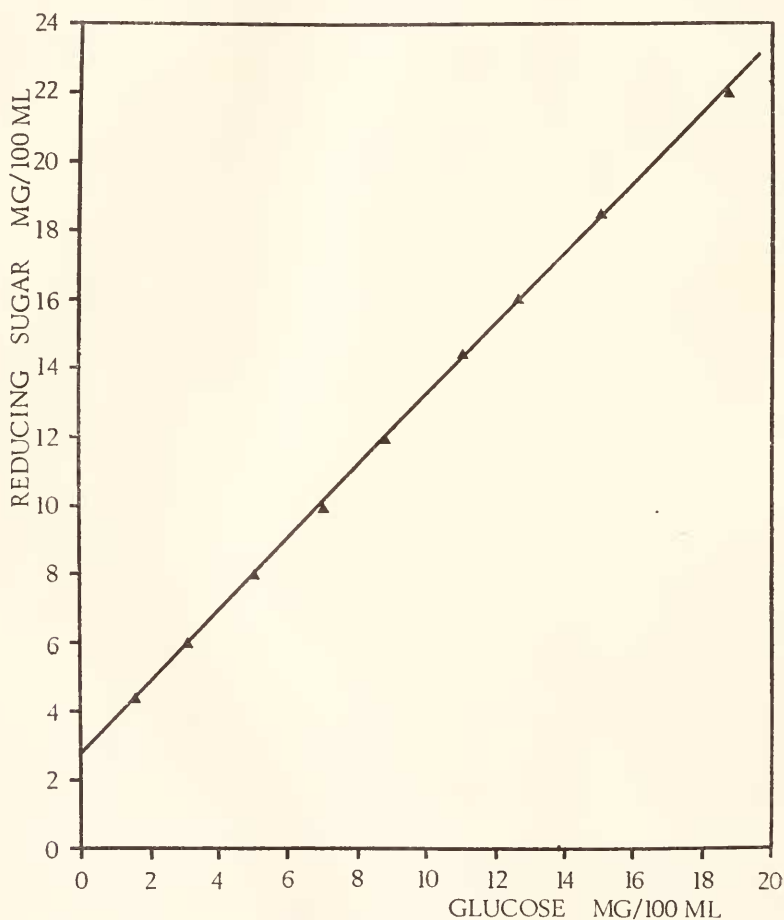


FIGURE 1. Relationship between blood glucose and reducing substances in the crab, *Cancer borealis*.

ways: (1) spray with 3% phthalic acid in 95% ethanol, viewed under UV light, (2) 0.5% benzidine in acetic acid-trichloroacetic acid-ethanol, and (3) AgNO_3 in acetone followed by 10% NaOH in 80% methanol (all methods in Dawson *et al.*, 1959).

Estimates of concentration in the spots were made by visual comparison with spots of known concentration and by chromatographing various sizes of samples to determine threshold of visibility with the detecting agents. Neither method is particularly accurate but estimates of the right order of magnitude are obtainable.

Reagents

The chemicals used were Fisher "Certified" and the carbohydrates for chromatography were obtained from Sigma Chemical Co. Ltd.

RESULTS

Mean levels of glucose and total reducing substances (RS) in the blood of *C. borealis* made at monthly intervals are given in Table I, together with the number of specimens (N) and the standard error of the mean (S.E.).

TABLE II

Blood glucose and reducing substances during the molt cycle of Carcinus maenas (mg./100 ml.)

| Stage | Drach (1939) | N | Glucose | S.E. | Reducing sugar | S.E. |
|----------------|--------------------------------|----|---------|--------|----------------|--------|
| Intermolt | C ₄ -D ₀ | 23 | 9.9 | (0.81) | 13.8 | (0.72) |
| Premolt | D ₁ -D ₄ | 11 | 12.2 | (1.37) | 17.1 | (1.41) |
| Very soft | A ₁ -A ₂ | 5 | 5.8 | — | 7.9 | — |
| Early postmolt | B ₁ -B ₂ | 2 | 5.7 | — | 8.0 | — |
| Postmolt | C ₁ -C ₃ | 19 | 8.4 | (0.90) | 13.5 | (1.07) |

An analysis of variance indicates that the variation in mean monthly glucose levels (1966) is significant at the 0.05 level. The differences in mean monthly reducing substance levels are generally not significant although a t-test between the July and August levels in males gives a significant result. In spite of appearances the difference between the sexes is not significant.

The individual glucose and reducing substance levels varied quite widely. Glucose ranged from 0.4 mg./100 ml. to 29.3 mg./100 ml. and the range for reducing substances was 3.3-29.5 mg./100 ml. The relationship between glucose and reducing substance is approximately constant. Figure 1 is a plot of mean glucose levels within 2 mg./100 ml. classes against the corresponding mean reducing substance levels. The line has a slope of 1.02 and an intercept of 2.9 mg./100 ml. when glucose is nil. The same values for slope and intercept can be obtained directly. The correlation coefficient between glucose and reducing substances, calculated directly from the 584 paired measurements, is highly significant ($R = 0.914$, $t = 54.22$ with 582 degrees of freedom). The analysis of covariance shows that this relationship between glucose and reducing substances

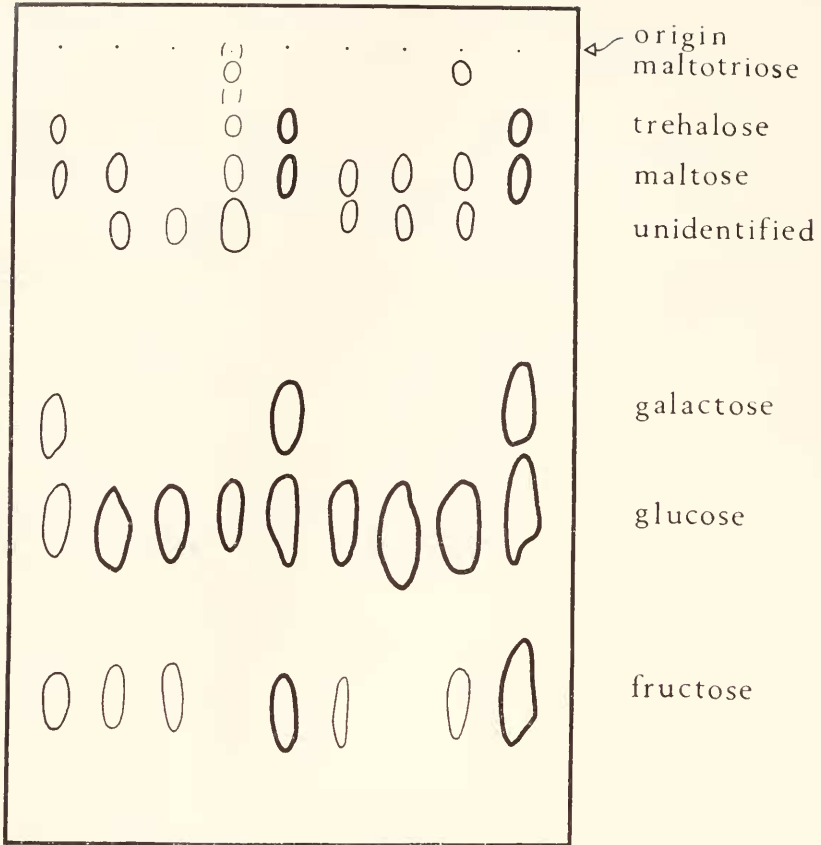


FIGURE 2. Paper chromatogram of blood sugars of *Cancer borealis*. Left, center and right, control mixtures of trehalose, maltose, galactose, glucose and fructose, 1.5 γ , 3.0 γ and 7.5 γ each, respectively (traced from photograph).

does not vary significantly from month to month, nor between the sexes, and it indicates the same level of significance for the variations in monthly mean glucose and reducing substance levels as did the analysis of variance already cited.

The crabs, *C. maenas*, were collected in September, 1967, in various molt stages. Intermolt and very early premolt, $C_1 - D_0$ of Drach (1939), were not separated but were treated together as a single intermolt stage which was recognized by color, hardness of shell, texture, color and consistency of the blood etc. Premolt, stages $D_1 - D_4$ of Drach, was recognized by darker color with blueness of chelipeds, development of new epicuticle and exocuticle and regeneration of damaged appendages. The earliest postmolt stage, stages $A_1 - A_2$ of Drach, was easily recognized by the non-calcified shells and the next postmolt stage, stages $B_1 - B_2$ of Drach, by the partially calcified exoskeleton. Postmolt, $C_1 - C_3$ of Drach, was recognized by the color and texture of the shell and blood. Table II shows blood glucose and reducing sugars in these general molt stages. These dif-

ferences are found by analysis of variance to be significant at the 0.05 level. No attempt was made to determine the significance, if any, of the differences between sexes, because there are too few observations to produce a reliable result.

A total of 67 specimens of *C. irroratus* were taken from lobster traps in May and June (1966), 31 males and 36 females. The mean glucose level for these was 8.1 mg./100 ml. (S.E. 0.23) and reducing substances 11.9 (S.E. 0.39); no difference in sex was apparent. No other determinations were made on this species (except chromatography, below) because *C. borealis* was readily available in much greater numbers in the areas where lobster traps were being set.

Chromatography of deionized blood samples (Figs. 2 and 3) revealed the occurrence of six sugars in the amounts estimated in Table III. Mobility of the sugars is expressed relative to glucose (R_g) because the solvent front was allowed to leave the paper. The bracketed percentage figure indicates the approximate number of samples in which each sugar was found. The estimated quantity is maximal in these samples. The unidentified carbohydrate was com-

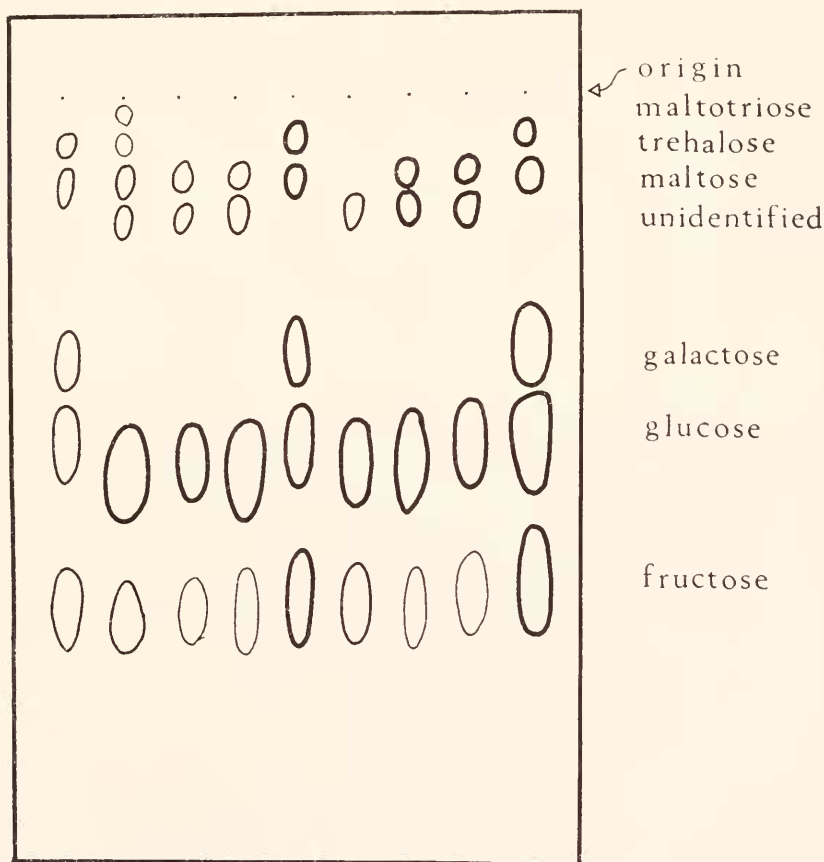


FIGURE 3. Paper chromatogram of blood sugars of *Carcinus maenas*. Controls as in Figure 2 (traced from photograph).

pared to maltose in estimating its concentration. The following criteria were used for identification of the sugars. Maltotriose: plot of \log_{10} Rg against number of hexose units places it on a straight line with maltose and glucose (McWhinnie and Saller, 1960) (Fig. 4). No maltotriose was available for direct comparison. Trehalose: same mobility as control spots, reinforcement of spots when trehalose added to sample, lack of reaction with phthalic acid or benzidine indicates non-reducing nature, appearance of spot with silver nitrate at same time as trehalose (this is due to the very strong alkali used, 10% NaOH). Maltose, glucose and fructose: mobilities, lack of proliferation when these sugars were added to blood samples, corresponding blood sugars show reducing activity and appear in same sequence and time intervals with AgNO_3 -NaOH spray (fructose-glucose-maltose). The mobility of the unidentified component suggests a disaccharide and its reaction with both phthalic acid and benzidine indicates a reducing group.

A total of ten female *C. borealis* was obtained carrying egg masses. On one of these the sponge was old; blood glucose and reducing substances levels were

TABLE III
*Estimated concentrations of blood sugars found in three species of crabs
with the frequencies of occurrence (%)*

| Sugar | Rg | <i>C. borealis</i> | <i>C. irroratus</i> | <i>C. maenas</i> |
|--------------|--------|----------------------|----------------------|------------------------|
| Maltotriose | (0.05) | trace (10%) | trace (30%) | 2 mg./100 ml. (30%) |
| Trehalose | (0.13) | trace (10%) | trace (10%) | 1.5 mg./100 ml. (10%) |
| Maltose | (0.27) | 2 mg./100 ml. (70%+) | 2 mg./100 ml. (70%) | 3 mg./100 ml. (30%) |
| Unidentified | (0.32) | 5 mg./100 ml. (100%) | 5 mg./100 ml. (100%) | 5 mg./100 ml. (100%) |
| Glucose | (1.00) | — (100%) | — (100%) | — (100%) |
| Fructose | (1.40) | 1 mg./100 ml. (60%) | 1 mg./100 ml. (30%) | 1.5 mg./100 ml. (100%) |

8.0 and 8.2 mg./100 ml., respectively, in this individual. The respective mean levels for the remaining nine were 12.36 (S.E. 1.18) and 14.47 (S.E. 1.19) mg./100 ml. Twelve females were found with bright orange-red blood and well developed ovaries. These were presumed to be preparing for spawning since the blood color was closely similar to that of the eggs and ovaries and this coloration never appeared in males. Mean glucose and reducing levels were 11.7 (S.E. 1.28) and 12.5 (S.E. 1.17) mg./100 ml. Three pairs of *C. maenas* were captured in copulation and blood was taken from all of the males and two females. No significant difference in blood sugar levels was found despite the ire and pugnacity of the animals (glucose 10.3, reducing substances 13.9 mg./100 ml.).

DISCUSSION

The blood glucose and reducing sugar levels reported here are comparable with those reported previously for decapod crustaceans but some interesting differences emerge. McWhinnie and Saller (1960) found in the crayfish *Orconectes virilis* that glucose levels averaged 3–4 mg./100 ml. and made up about 20–25% of the total reducing substances (by Folin-Wu method). Earlier McWhinnie and Scheer (1958) found the same relationship between glucose and total carbohydrate

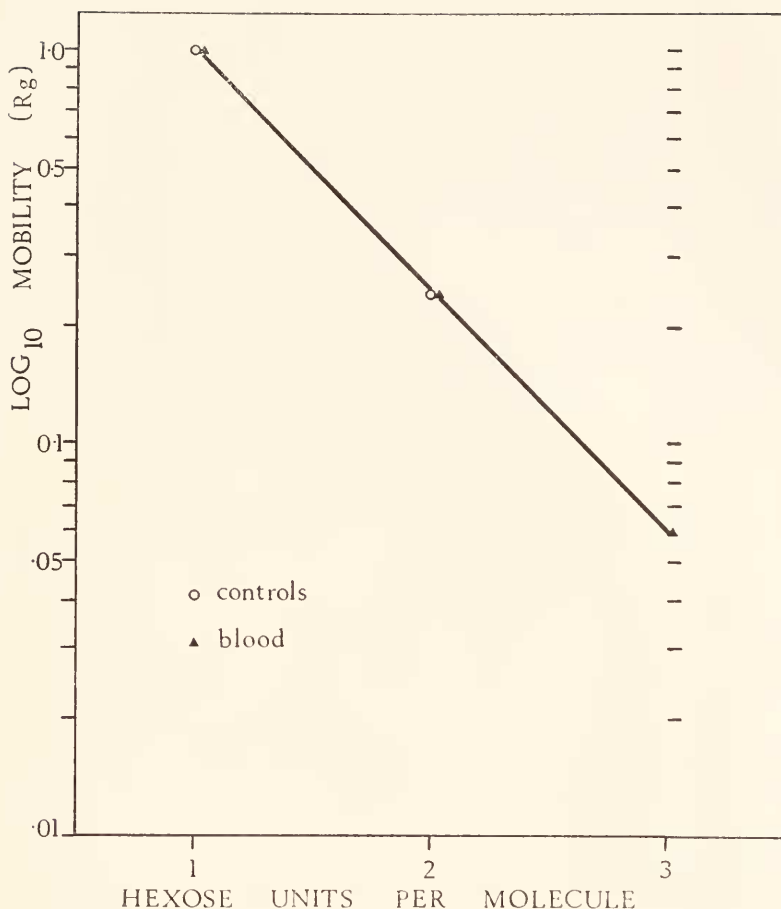


FIGURE 4. Identification of presumed maltotriose by plotting molecular size and \log_{10} mobility (after McWhinnie and Saller, 1960).

(by anthrone method) in the crab *Hemigrapsus nudus*. More recently Dean and Vernberg (1965a, b) using the same methods as McWhinnie and Saller (1960), found the same relationship in several species of crabs and stated specifically that no matter what the glucose level, its ratio to total reducing substances was constant. This is clearly not the case in *C. borealis* where glucose is the variable blood sugar and the non-glucose component remains approximately constant at about 3 mg./100 ml. In the lobster, *Homarus americanus*, this same relationship has been found, variable glucose levels and approximately constant non-glucose around 4.4 mg/100 ml. (Telford, 1968a). In the special case of females carrying eggs there is evidence of significant differences between them and other females (0.01 level by *t*-test). In Table IV these and similar data from Dean and Vernberg (1965b) are compared.

A similar series of changes in glucose levels evidently occurs in the two species but according to the data for *C. sapidus* another reducing substance also undergoes

the same changes. No attempt was made to identify this substance. Dean and Vernberg (1965b) appear to have used a heat-coagulated blood for their reducing substance determination. This method does not completely deproteinize the sample and leaves most or all of the non-carbohydrate reducing matter in the filtrate. These interfering substances may have masked the true relationship between glucose and reducing sugars in the studies with heat-coagulated blood.

The amount of color produced by several sugars with the Folin-Malmros method used in this study was determined and expressed as a percentage of the color given by the same amount of glucose. Trehalose, as expected, gave no reaction; maltose gave 60% of its glucose equivalent, fructose 104%. The estimated amounts of sugars other than glucose found in chromatograms (Table III) are the maximum levels found. Assuming that the unknown reducing disaccharide would react like maltose (lactose, for example, gives about 55%), then the total non-glucose carbohydrate component of the reducing substances would have a maximum value of about 6 mg./100 ml. The estimated normal level is closer to 2 mg./100 ml. or,

TABLE IV

Glucose and reducing substances levels in C. borealis and C. sapidus carrying egg sponges

| Species | N | Glucose mg./100 ml. | S.E. | Reducing mg./100 ml. | S.E. | Gluc/Red × 100 |
|--|----|------------------------|--------|-------------------------|--------|-------------------|
| <i>Cancer borealis</i> | | | | | | |
| no eggs | 43 | 6.59 | (0.47) | 10.31 | (0.55) | 64% |
| new eggs | 9 | 12.36 | (1.18) | 14.47 | (1.19) | 85% |
| old eggs | 1 | 8.00 | — | 8.20 | — | 97% |
| <i>Callinectes sapidus</i> | | | | | | |
| no eggs | 7 | 18.47 | (2.36) | — | — | 20–25% |
| new eggs | 7 | 37.61 | (2.77) | — | — | 20–25% |
| old eggs | 9 | 10.52 | (1.03) | — | — | 20–25% |
| <i>C. sapidus</i> data from Dean and Vernberg (1965b) | | | | | | |

about 60–65% of the non-glucose component. In another study of the lobster, *Homarus americanus*, the non-glucose part of the reducing substances was estimated to be about 50% carbohydrate (Telford, 1968a, 1968b).

In the study of the lobster referred to above a cycle of changes in blood sugar levels during the molt cycle was found in a group of 800 animals. The apparent cycle found here in *C. maenas*, although the number of specimens is much lower, is closely similar. Molting of crabs on the coast of Maine occurs principally in the late summer and fall. The seasonal changes in *C. borealis* probably reflect the molting cycle with glucose increasing in premolt (August–September) and dropping in postmolt. The differences here are not clearly defined because the different molt stages were not separated, the monthly samples being made up of a changing proportion of premolt and other animals. In previous studies such a cycle of changes has not been found although expected (McWhinnie and Scheer, 1958; Scheer, 1959; McWhinnie and Saller, 1960).

As a regular component of crustacean blood fructose has not previously been reported. It was reported as occasionally occurring in *H. americanus* (Telford,

1965; 1968a, 1968b) but it was not found by either McWhinnie and Saller (1960) or Dean and Vernberg (1965a). It has been reported in some insects (Levenbrook, 1950). No sign of the curious "galactan derivative" of McWhinnie and Saller (1960) was found, nor of galactose. Maltotetrose has often been reported by other workers but no spot corresponding with its probable position could be found with the detection reagents used here, nor by examination under UV light when tetroses should fluoresce. Following molting the blood of *C. maenas* has only traces of sugars other than glucose; the oligosaccharides are severely depleted as in lobsters at this stage (Telford, 1968a).

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SUMMARY

1. Blood glucose levels in the crab *Cancer borealis* ranged from 0.4 mg./100 ml. to 29.3 mg./100 ml. with a mean value of 8.6 mg./100 ml. In the same animals blood reducing substances were in the range 3.3–29.5 mg./100 ml. with a mean of 11.6 mg./100 ml. Blood glucose and reducing substances in the other two species of crabs tested, *Cancer irroratus* and *Carcinus maenas*, were in the same ranges.

2. Changes in the blood glucose level account for most of the variations in reducing substances; the other components remain approximately constant, at about 3 mg./100 ml. The relationship between blood glucose and reducing substances is thus a simple straight line one.

3. Variations of blood sugar levels during the molt cycle were found in *Carcinus maenas* and probably occur also in *Cancer borealis*. Qualitative changes in blood sugar composition also occur.

4. Significant changes also occur in *Cancer borealis* females carrying eggs and at this time the relationship between blood glucose and reducing substances changes, the two values converging as the egg mass ages.

5. Paper chromatography of the blood of these three species of crabs shows the presence of glucose, fructose, maltose, an unidentified reducing disaccharide and occasional traces of maltotriose and trehalose.

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